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Characterization of Dimethyl sulfoxide Reductase from Brewing Yeast

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Abstract

Dimethyl sulfoxide (DMSO) Reductase is present in all brewing strains tested, but is more readily detectable using activity stains on polyacrylamide gels than through direct spectrophotometric assay of crude extracts. The enzyme is detectable at higher activities in yeast cultivated at lower temperatures and this is because of increased expression of the primary gene that codes for the enzyme in yeast, MXR1. DMSO somewhat increases enzyme expression, notably in lager yeast by up to 70%, whereas methionine suppresses it. Both ale and lager strains produce enzymes of approximately 100,000 in molecular weight, but ion exchange chromatography divides the activity for both ale and lager yeasts into two fractions with distinct properties (heat tolerance, pH optimum, kinetic parameters).

Key words: Dimethyl sulfoxide; enzyme; gene expression; reductase; temperature

Introduction

One of the sources of dimethyl sulfide (DMS), which contributes a character variously described as canned corn, ketchup, marine or peeled parsnip to certain beers especially lagers (1), is dimethyl sulfoxide (DMSO), which is reduced to DMS by brewer's yeast (2). It was shown that the enzyme that effects the reduction of DMSO is a methionine sulfoxide reductase (EC 1.8.4.13) to which reducing power is fed by the thioredoxin/thioredoxin reductase couple (3). Mutants of yeast devoid of methionine sulfoxide reductase were incapable of producing DMS (4). Studies revealed that up to 80% of the DMS in a lager beer originated from DMSO (5). Various parameters appear to play a role in dictating the extent to which yeast can reduce DMSO and these include temperature, with lower fermentation temperatures yielding higher degrees of DMS production by yeast (6), and the availability of assimilable nitrogen, with more DMSO reduction at lower levels of free amino nitrogen (7). The control of DMS in beer was recently reviewed, wherein it was highlighted that the thermal decomposition of S-methylmethionine is a key source of DMS in beer (8).

In the present work we have taken a fresh look at DMSO Reductase, with a view to investigating various previously incomplete aspects of this system, including its molecular nature and its kinetic properties.

Materials and Methods

Materials

Unless otherwise indicated all chemicals were from Wako Pure Chemical Industries Ltd., Osaka, Japan

Yeast strains

The yeast strains studied are listed in Table 1.

Media

YPD medium (glucose, 2%; yeast extract, 1%; peptone, 2%) was used for cultivation of yeast. Glucose-salts medium was prepared according to Wainwright (9). Maltose-salts medium was prepared in the same manner but substituting 5% maltose for glucose in this medium. Malt extract medium comprised malt extract (Muntions Dry Malt Extract, extra light) dissolved in deionized water to a measured strength of 10° Plato.

Cultivation of the yeast

Yeast was pre-cultured in YPD medium at 30 °C for 24 hr statically. Cells were counted microscopically using a Thoma-Zeiss hemocytometer .

The media (300mL) in Erlenmeyer flasks (400 mL capacity) were sterilized at 121°C for 15 min prior to cooling to 15°C and inoculation with yeast (10^6 cells/ml) prior to an incubation time of 72h without shaking.

Extraction of cells

At the end of the growth period, media (10mL) were centrifuged at $10,000\times g$ for 20min at 4°C.

The collected yeast was re-suspended in 50mM phosphate buffer (pH 7.0) containing 2mM EDTA, 2mM 2-mercaptoethanol and 0.5% sodium chloride in screw cap tubes (2mL volume, AZ ONE Osaka Japan). The cell suspensions were disrupted in a cell crusher (#0061173-000, TAITEC Co. Saitama, Japan) at 4 °C. Crushed cell suspensions were centrifuged at $10,000\times g$ for 20min at 4°C. The supernatant represents the crude enzyme extract.

Preparation of reduced thioredoxin

Thioredoxin was from recombinant yeast (#47759000, Oriental Yeast Co. Ltd). Thioredoxin was reduced (where required) using dithiothreitol (DTT; 10). Excess DTT was removed using Micro Bio Spin (Bio Rad, Hercules CA, U.S.). Procedures were conducted in duplicate.

Assay of DMSO Reductase in crude extracts

DMSO reductase was assayed according to the method of Bamforth (3). Assays (10.0 mL) contained 2 mM DMSO, 1mM NADPH (Nacalai Tesque Inc. Kyoto, Japan), 10 μ g/mL thioredoxin (Oriental Yeast Co.) and 5 Unit/mL of thioredoxin reductase (rNRT, Oriental Yeast Co.) in 200mM phosphate buffer (pH 7.0). The mixtures were held in 20mL vials for headspace GC (6890N network GC systems, Agilent Technologies, Santa Clara, CA, US) and were closed by crimp top seals. Crude enzyme extracts (0.4mL) were added to the vial by syringe and incubation was at 30°C for 12 min. After reaction, 1M sodium hydroxide (1ml) was injected to stop the reaction and samples were analyzed by GC-FID using ethyl methyl sulphide (EMS; final concentration 100ppm) as internal standard. EMS was added to the reaction mixtures by syringe. GC analysis was using a headspace sampler (G1888, Agilent Technologies) and GC system (G1888 Agilent Technologies) using capillary column (Agilent J&W HP INNO-WAX, #19091N-133, Agilent Technologies). The conditions are summarized in Table 2. A standard curve was prepared over a range of 0.005ppm to 2.0ppm DMS in 3% ethanol. Procedures were conducted in triplicate.

Assay of Protein

The protein concentration was assayed using the method of Bradford (11). The method was calibrated using bovine serum albumin. Procedures were conducted in duplicate or triplicate.

Detection of DMSO reductase by Native PAGE

Samples were separated on a 12.5% uniform gel (E-T12.5L; Atto Co. Tokyo Japan).

Electrophoresis was conducted as described by Laemmli (12). Crude extracts (10 μ L) were added to 0.5 M Tris-HCl pH6.8 buffer (10 μ L) containing 0.002% bromophenol blue and 10.0% glycerol prior to electrophoresis. Following electrophoresis the gel was soaked in a reaction mixture (50mL) comprising 2 mM DMSO, 1mM NADPH, 10 μ g/mL of thioredoxin and 5 Unit/mL of rNRT in 200mM phosphate buffer, pH 7.0 at 30°C for 30min (13). White was reversed to black in the photograph was reversed by software, Microsoft office power point 2013. The gel was also stained using Coomassie Brilliant Blue using EzStain Aqua (#AE-1340, Atto Co.) to detect protein. Protein was detected at 365nm by UV illuminator (#GDS-7900, UVP inc., Upland, CA. U.S.).

Growth of the yeast

Cells were incubated in 300mL of glucose-salt medium (500mL vol. Erlenmeyer flask) at 8, 16 and 20°C for 96 h statically under slight anaerobic condition. And growth was monitored by measuring optimum density at 660 nm using a spectrophotometer (Nano drop 2000, Thermo Scientific, Waltham MA).

Purification of DMSO reductase from yeast

Lager yeast and ale yeast were cultivated in 10L of glucose-salt medium containing 100 mg/L DMSO. After centrifugation, yeast cells were harvested, with yields for the lager yeast of 5.4g dry weight and for the ale yeast of 7.7g dry weight. The enzymes were extracted with 50mM phosphate buffer (pH 7.0) containing 2mM EDTA, 2mM 2-mercaptoethanol and 0.5% sodium chloride with glass beads by pestle and mortar (see above). The extracts were precipitated to between 60–80% saturation with ammonium sulfate. The precipitate was re-dissolved in 10 mL of 50 mM phosphate buffer, pH 7.0, containing 2mM 2-mercaptoethanol. The solution was dialyzed overnight at 4 °C against 1 L of 50 mM phosphate buffer, pH 7.0, containing 2mM EDTA and 2mM 2-mercaptoethanol. Ion exchange chromatography was performed on the enzyme solution using a column (25 mm × 300 mm) of DEAE gel (Macro-Prep support; Bio-Rad Laboratories). The protein was eluted using a 0–1 M NaCl linear gradient. The eluent flowed at 3 mL per min. The enzyme solution was collected in 4 mL fractions. Samples containing the highest level of activity were pooled and re-precipitated using 80% saturation of ammonium sulfate. The precipitate was re-dissolved in 2 mL of 50 mM phosphate buffer, pH 7.0. The molecular weight of the enzyme was assessed using gel filtration chromatography (10

mm × 350 mm, P-100 gel; Bio-Rad Laboratories). Molecular weight standards were myosin, 200 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa and trypsin inhibitor, 21.5 kDa. Phosphate buffer, 50 mM, pH 7.0 was the eluant at a flow rate of 3.8 mL/min and 1 mL fractions were collected. In all column chromatography eluting protein was monitored by absorbance at 280nm by spectrophotometer (Nano drop 2000).

Assay of DMSO reductase during purification

DMSO reductase was assayed using a slight modification of the method of Bamforth (3). Enzyme solution (10 μ L) was added to a substrate mixture (200 μ L) comprising 1mM NADPH, 2mM DMSO, 10 μ g/mL thioredoxin, 5 Unit/mL rNRT, 10mM EDTA and 10mM dithiothreitol in phosphate buffer (50mM, pH 7.0) using 96 well microplate (AGC Techno Glass Co., Shizuoka Japan). The decreasing NADPH concentration was assayed by measuring absorbance at 340 nm using a microplate reader (Multiskan FC, Thermo Scientific). One unit of DMSO reductase represents that quantity which catalyzes the oxidation of 1 mM NADPH per minute.

Extraction of total RNA

Total RNA was extracted by a Tissue Total RNA Mini Kit (Favorgen Biotech Corp., Ping-Tung, Taiwan). Cells, harvested from 2mL of cultured medium, were extracted according to the protocol in the kit. Procedures were conducted in duplicate.

Synthesis of cDNA from the total RNA

cDNA was synthesized from purified total RNA using a Prime Script RT reagent kit with gDNA Eraser (Perfect Real Time, Takara Bio Inc. Otsu Shiga Japan). Purified total RNA (500ng) was added to gDNA Eraser Buffer (2µl), gDNA Eraser (1µl) and they were adjusted to 10 µL by DEPC treated water. After the addition of 4µl of Prime-Script Buffer, 1µl of PrimeScript RT Enzyme Mix and 4µl of RNase free water, samples were incubated at 37°C for 15min, and then 85°C for 5 sec.

Real-time RT-PCR analysis

Real-time RT-PCR analysis used a SYBR *Premix Ex Taq*TM II (Tli RNaseH Plus, #RR820A) by Mx3000P QPCR System (Agilent Technologies). Expression levels of DMSO reductase were assayed by primers as MXR1 (Forward; 5'-TTGCGGAGG TTTTACAAGTATC-3' and Reverse; 5'-GCATCTGA ATGAGCGAACAA-3'), or MXR1-CA (Forward; 5'-TGCGTGCCCTACTCACTATTT G-3' and Reverse; 5'-AATGGCCTTGCCCTTCC-3'). Expression levels of housekeeping gene as control were by primers as TAF10 (Forward; 5'-CCAGGATCAGGTCTTCCGTA-3' and TAF10 Reverse 5'-CAACAGCGCTACTGAGATCG-3') (14). The data was calculated by the $2^{-\Delta\Delta C_T}$ method (15). Procedures were conducted in duplicate.

Measurement of optimum pH

Optimum pH was assayed over the pH range 4.0–8.0 using 50 mM phosphate-citric buffer or phosphate buffer in the standard assay (see above).

Evaluation of heat tolerance

Each purified enzyme solution was incubated in covered tubes at 40°C, 50°C, 60°C, 70°C, 80 °C, or boiling water for between 10 and 120min before rapidly cooling in ice prior to assay. The control sample was held at 4 °C. Procedures were conducted in duplicate.

SDS-polyacrylamide gel electrophoresis

Samples were separated on a 12.5% uniform gel (E-T12.5L; Atto). Electrophoresis was conducted as described by Laemmli (12) with the following modifications: samples (0.01 mL) were added to 0.01 mL of sample buffer (EzApply, AE-1430, Atto) and then heated at 100 °C for 3 min. Samples were added at 10 µl in each well. The gels were run at 20 mA with 1.5 mm gel thickness. Molecular weight standards were either protein standard mixture (Bio-Rad

Laboratories Inc) or Ez Standard (Atto Corp. Cat.# AE-1440). The gel was stained with 0.25% Coomassie Brilliant Blue R-250 or using the silver stain method (Silver Stain II Kit Cat. #291-50301; Wako).

Determination of kinetic parameters

The substrate kinetic analysis was conducted according to Dalziel (16) using the standard assay with varying substrate concentrations: DMSO was used at either 0.625mM, 1.25mM, 2.5mM, 5.0mM or 10.0mM and thioredoxin at 25mg/mL, 12.5 mg/mL, 6.25 mg/mL or 3.125 mg/mL.

Various electron donors for DMSO reductase

DMSO reductase activity was assayed replacing the thioredoxin system with either 10 μ M or 100 μ M reduced glutathione or 10 μ M or 100 μ M cysteine.

Calculation of standard deviation

Statistical analyses were conducted using software (Microsoft Office 2003 Excel; Microsoft Corp., Redmond, WA).

Results and Discussion

DMSO Reductase in a range of yeast strains growing on glucose or maltose

Yeast was cultivated on glucose-salts medium, maltose-salts medium or malt extract and the cell extracts assayed for DMSO reductase activity (Fig 1). The enzyme was not detected in all yeast strains. Where it was detected the highest levels were generally detected in the glucose-salts medium, with rather less detected when maltose was the main carbon source, either in a simple salts medium or in a medium based on malt extract, which will have a composition close to brewer's wort. This is in contrast to the findings of Samp et al (17), who found higher extents of DMS production when maltose was the adjunct sugar. Younis and Stewart (18), however, found that various volatiles produced by yeast, notably esters and higher alcohols (they did not investigate DMS), were generally produced in lesser quantities with maltose-supplemented worts as opposed to glucose-supplemented worts, although they did not firmly conclude why this is the case.

When the enzyme was measured by staining on polyacrylamide gels, all strains except the laboratory strain displayed detectable DMSO reductase activity (Fig 2). The lager strains did appear to give bolder bands of staining. It is suggested that although levels of the enzyme in the majority of the strains tested are rather low, the enzyme is present as detected on the more

sensitive electrophoresis-based system. It is also possible that there are inhibitors present in the extracts that cause the diminution of activity as detected in the standard assay, but which are not able to interfere with the detection system on the gels as they are not co-migrating with the protein. Equally it is possible that the reducing power from the thioredoxin/thioredoxin reductase couple is being diverted to other electron recipients in the assay mixture. Irrespective of the explanation, it draws attention to the possible shortcoming of assay of enzymes such as DMSO reductase in crude extracts.

The impact of temperature on yeast growth and levels of DMSO reductase

Neither the selected ale strain (US-O5) nor the selected lager strain (2007 Pilsen) selected grew well on glucose-salts medium at 8°C (Fig 3). It will be noted that we deliberately used a relatively low inoculation rate and avoided oxygenation of the cultures.

When sampled after 72h, the yeast contained DMSO reductase in inverse proportionality to growth temperature (Fig 4). This was especially marked for the lager strain. It is also seen that whilst there is a significant quantity of DMSO reductase in yeast that has not been exposed to DMSO, the level of enzyme detected increases with the amount of DMSO added to the medium, again notably with the lager strain (Fig 5).

Gene expression

To assess whether the level of DMSO reductase expressed under various conditions is a function of differences in the level of gene expression, we ascertained the extent to which the primary gene coding for this enzyme, MXR1 (19), is expressed, together with the extent of expression of a related gene MXR-CA, which is only present in lager strains (20; Fig 6). The first of these genes is expressed at an increased rate at the lower temperatures, however the converse was observed with MXR-CA. Expression of both genes was increased in the presence of DMSO but the expression of MXR1 was lowered in the presence of methionine (Fig 7). The data suggests that higher levels of DMSO reductase at reduced temperature are a consequence of increased expression of the enzyme from the MXR1 gene. Hansen et al (19) concluded that the MXR1 gene was much the more significant for DMS production in lager strains. Curiously, like Hansen et al (19), we find methionine lowers the transcription of the enzyme. This conflicts with the findings of Gibson et al (7), who showed that methionine boosted the reduction of DMSO. This would suggest that the increase reduction of DMSO in the presence of methionine as reported by Gibson et al (7) was not related to gene expression but to some other aspect of metabolism, perhaps a lessened channeling of thioredoxin reducing power into other functions. It should be noted that although there are two genes coding for DMSO reductase in lager strains, only a solitary band of enzymic activity is detected by polyacrylamide gel electrophoresis (Fig 2).

Partial purification and properties of DMSO reductase

Notwithstanding this solitary band of DMSO reductase witnessed on polyacrylamide gels, partially purified DMSO reductase from both the lager and the ale strain in each case reveals two separate peaks of activity were upon ion exchange chromatography (Fig 8). It would be tempting to suggest, based on the concentration of salt at which the enzymes are eluted, that the predominant activity from yeast (isomer II) is equivalent to isomer I from the ale strain. However their properties are very different (see later). The lager strain contains a more cationic isomer while the ale strain has a more anionic isomer.

As gauged by elution on gel permeation chromatography (Fig 9) it would appear that each of the isozymes is of a similar molecular size. Upon polyacrylamide gel electrophoresis (Fig 10 a), a molecular weight for the enzyme of ca. 40,000 is indicated, while gel permeation chromatography (Fig 10b) indicates a molecular weight of 100,000, suggesting that the enzyme is a dimer.

That there are molecular differences between the various forms of DMSO reductase is suggested by differences in their tolerance to heat (Fig 11) and pH activity profiles (Fig 12). Rowe et al (21) showed an intracellular pH for brewing yeast in the range 5.9 – 6.4, thus it will be recognized that all of the enzymes reported here have optima at or around that range. Detailed 2-substrate kinetic analysis of the enzymes shows distinct differences in the key parameters of Michaelis Constant and Maximum Velocity (Table 3). In the case of both the ale and lager strain, Enzyme II has the greater affinity of both substrates, with lager yeast enzyme II having highest affinity for DMSO.

Finally we investigated the ability of the enzymes to function with alternative sources of reducing power (see Fig 13, 14). In all instances the presumed ‘natural’ source of electrons (reduced thioredoxin) is the preferred donor, however glutathione and cysteine are capable of providing reducing power although there are differences between the four enzymes.

A comment on the possible significance of DMSO in wort other than as a precursor of DMS

Kwak et al (22) refer to two major methionine sulfoxide reductase families, A and B, and claim that only the former is capable of reducing DMSO. They assert that DMSO inhibits both enzymes but by different mechanisms. The A enzyme is competitively inhibited, as would be expected from a competing substrate. Inhibition is much more powerful for the A enzyme. It is unclear whether the two enzymes reported in the present study reflect the A and B families reported by Kwak et al, though it seems unlikely as both of the fractions reported here are DMSO reductases. The investigations of Kwak were performed in the context of DMSO interfering with the ability of methionine sulfoxide reductase to function in a protective manner in the organism: this potential negative impact of the presence of DMSO, which is in all worts (6), on the health of brewing yeast has not hitherto been considered. However it was shown that the affinity of the reductase for methionine sulfoxide is substantially greater than for DMSO (23). Nonetheless it is an area of inquiry that is worthy of pursuit. Both methionine sulfoxide and DMSO are present at significantly higher concentrations in worts made from more heavily kilned and roasted malts (24).

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Table 1

Yeast strains for assay of DMS during fermentation/growth in the medium

Lager yeast (all from Wyeast Laboratories, Hood River, Oregon, US)	2000 Budvar Lager, 2007 Pilsen Lager 2035 American Lager 2206 Bavarian Lager Saflager W34/70
Ale yeast (all from Charles Faram Co Ltd, Malvern UK)	US-05 Ale S-04 Ale S-33 Ale
Wine yeast	W-3
Sake yeast	Sake yeast K-7
standard strain	<i>S. cerevisiae</i> NRIC1560

Table 2

Gas chromatographic analysis of DMS

Headspace sampler	
Sample incubation	70°C, 10 min
Loop temperature	85°C
Transfer line temperature	95°C
Thermostat time:	20 min
Vial pressure:	15 psi
Cycle time:	13.5 min
GC	
Injection temperature	140°C, 5:1 split ratio in split mode
Helium carrier gas flow rate:	1.3 mL/min, constant flow mode
Oven temperature program:	Rising 5°C/min from 50°C to 100°C
FID temperature	250°C
Capillary column	HP INNO-WAX (0.25 mm X 30 m, Agilent J&W)

Table 3

		Φ_0 (s)	Φ_1 (mM.s) (DMSO)	Φ_2 (μ M.s) (Thioredoxin)	$1/\Phi_0$ (s ⁻¹) Maximum Velocity	Φ_1/Φ_0 (mM) Km for DMSO	Φ_2/Φ_0 (μ M) Km for Thioredoxin
Ale	I	10.5	31.2	17.3	0.09	2.97	1.64
	II	24.9	62.1	15.8	0.04	2.50	0.64
Lager	I	23.0	24.2	37.1	0.04	1.05	1.61
	II	11.9	4.7	7.7	0.08	0.39	0.64

Fig.1

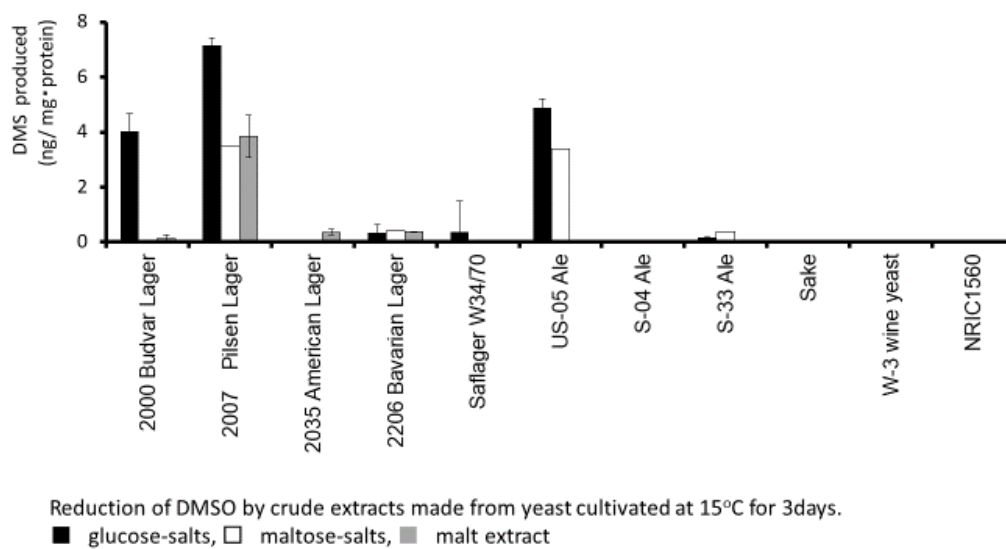
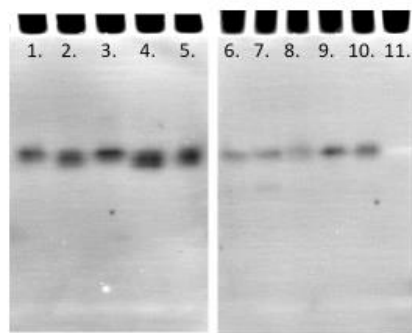
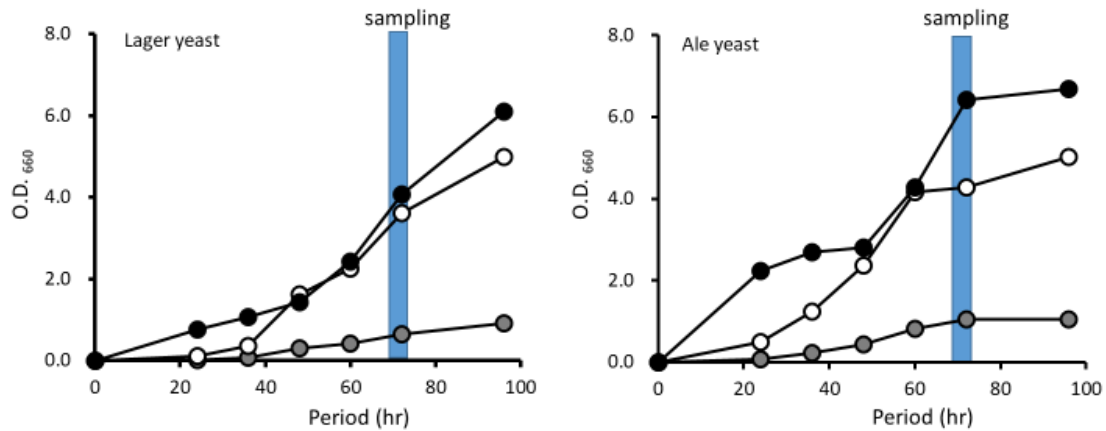


Fig.2



DMSO reductase activity on native gel electrophoresis. The crude enzyme solutions examined were in lanes as shown: 1. 2000 Lager; 2. 2007 Lager; 3. 2035 Lager; 4. 2206 Lager; 5. W34/70 Lager; 6. US-05 ale yeast; 7. S-04 ale yeast; 8. S-33 ale yeast; 9. Sake yeast No7; 10. Wine yeast W-3; 11. *S. cerevisiae* standard strain NRIC 1560.

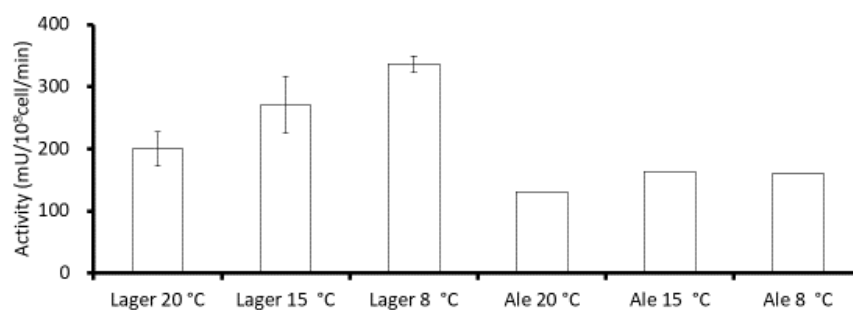
Fig.3



Growth of lager and ale yeast on glucose-salt yeast at 8, 16 and 20 °C. The yeasts were 2007 Pilsen lager yeast and US-05 ale yeast

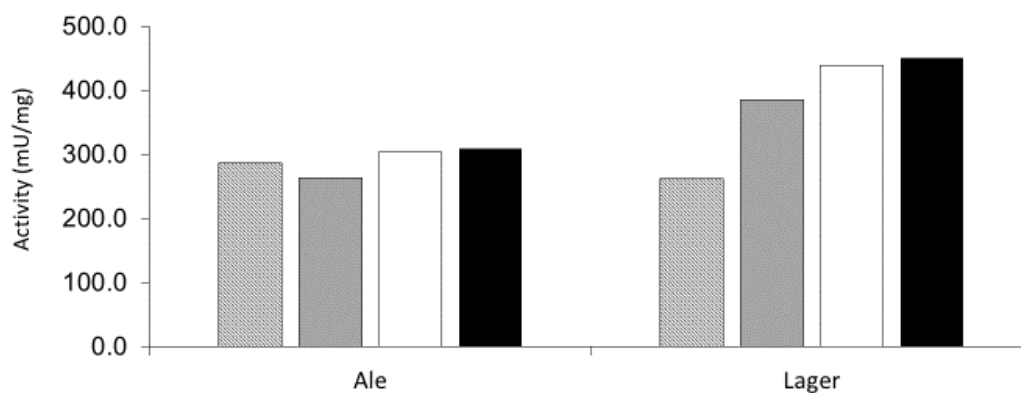
● 8 ° C, ○ 16 ° C, ● 20 ° C,

Fig.4



DMSO reductase in crude extracts of yeast grown on glucose-salts medium for 72h at different temperatures. The yeasts were 2007 Pilsen lager yeast and US-05 ale yeast.

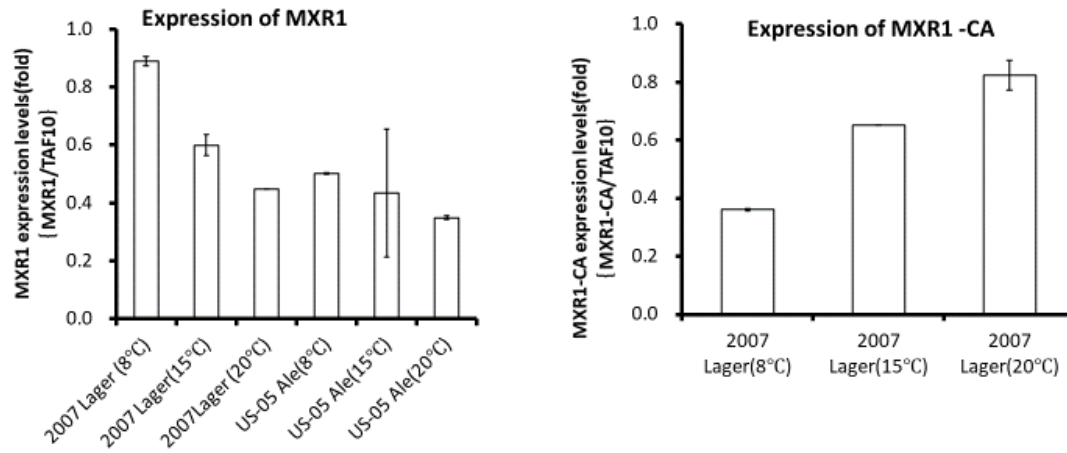
Fig.5



DMSO reductase in crude extracts of yeast grown on glucose-salts medium for 3 days in medium containing different levels of DMSO.

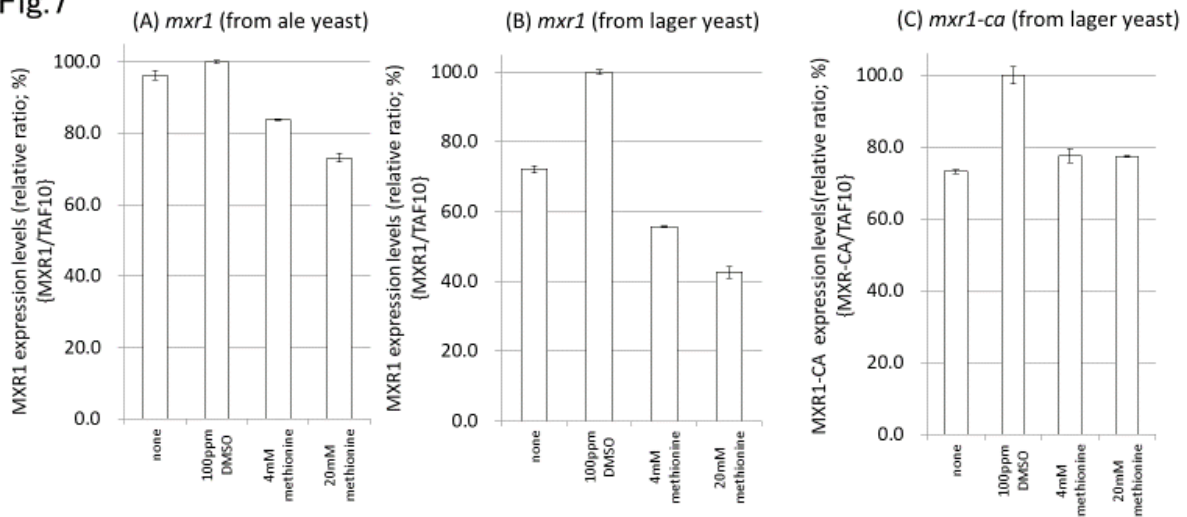
0 ppm, 25 ppm, 50 ppm, 100 ppm

Fig.6



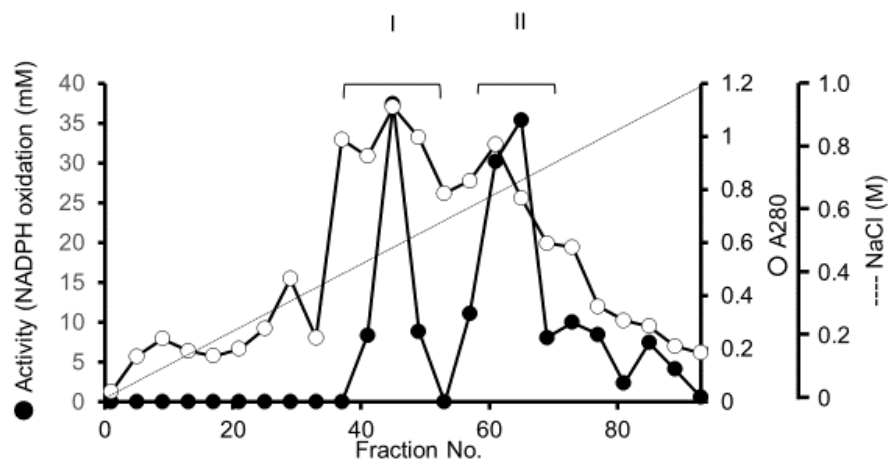
Expressions of MXR1 and MXR-ca in lager yeast and MXR1 in ale yeast, cultivating at 8, 15 and 20 ° C for 3 days

Fig.7



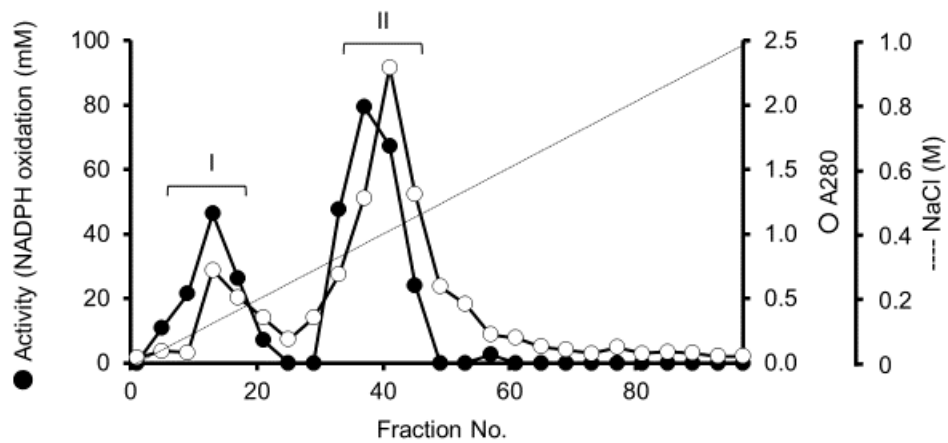
Expression of *mxr1* and *mxr1-ca* of Lager and Ale yeast grown at 8°C on glucose salts medium in the presence of added DMSO or methionine

Fig.8a



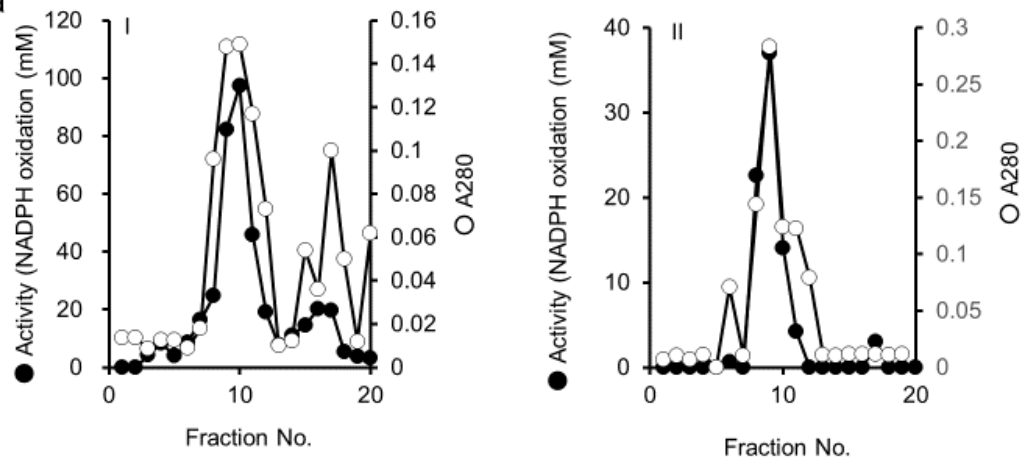
Ion exchange chromatography of DMSO reductase from Ale yeast. The dotted line indicates NaCl.

Fig.8b



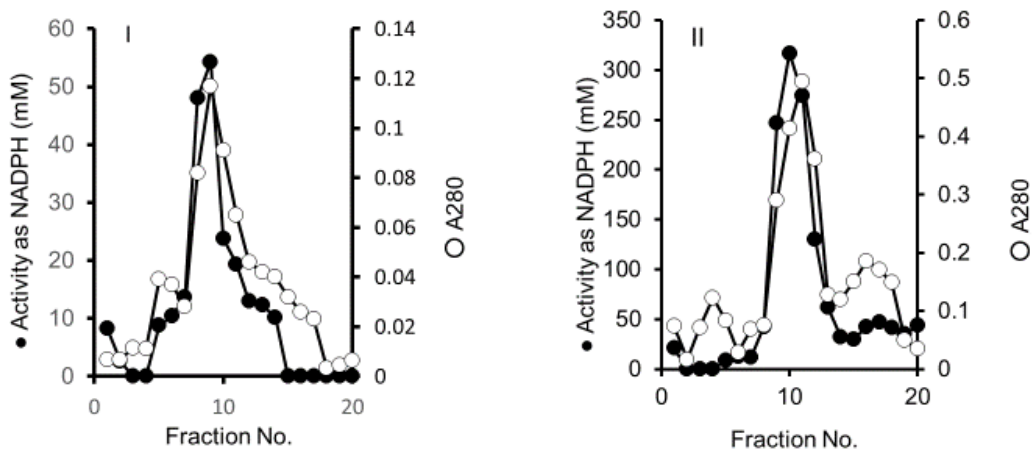
Ion exchange chromatography of DMSO reductase from Lager yeast. The dotted line indicates NaCl.

Fig.9a



Gel permeation chromatography of DMSO reductase I and II from Ale yeast.

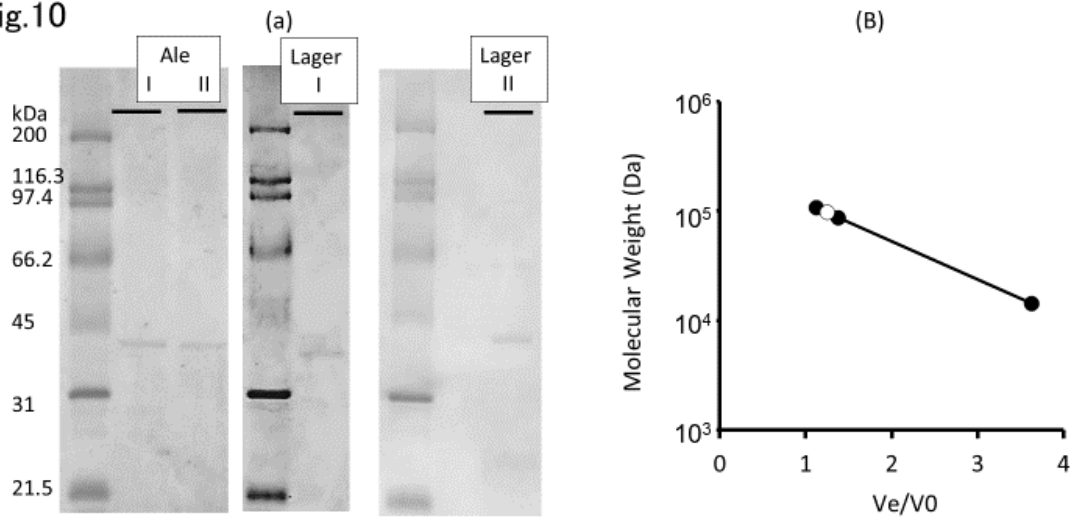
Fig.9b



Gel permeation chromatography of DMSO reductase I and II from Lager yeast.

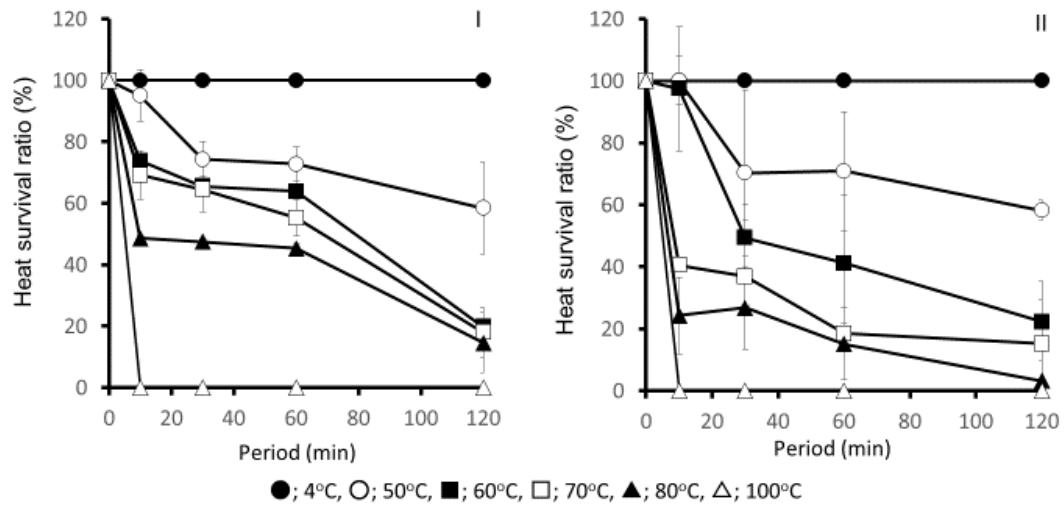
o)

Fig.10



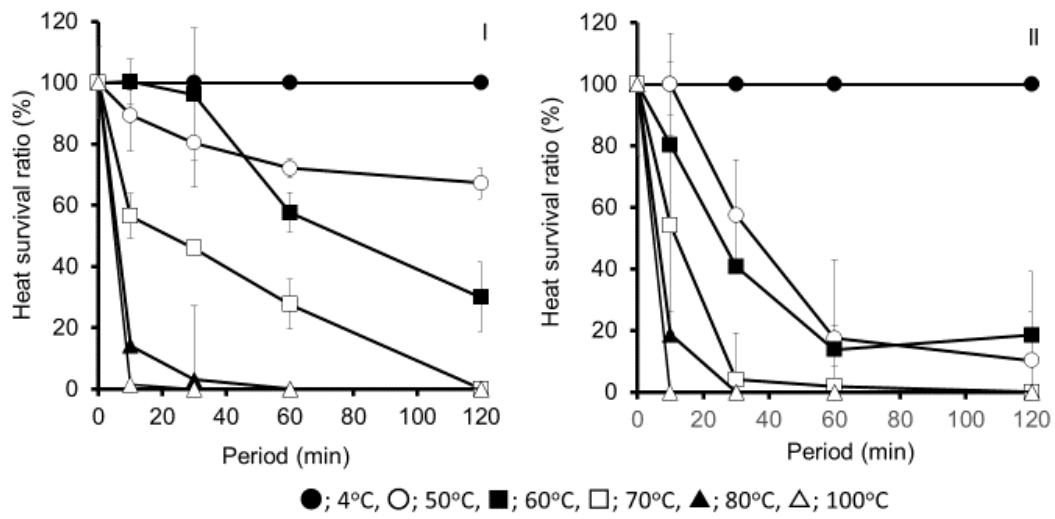
SDS-PAGE (a) and molecular weight by gel permeation (b) of DMSO reductase. In (b) the open circle is the enzyme while the closed circles are reference standards

Fig.11a



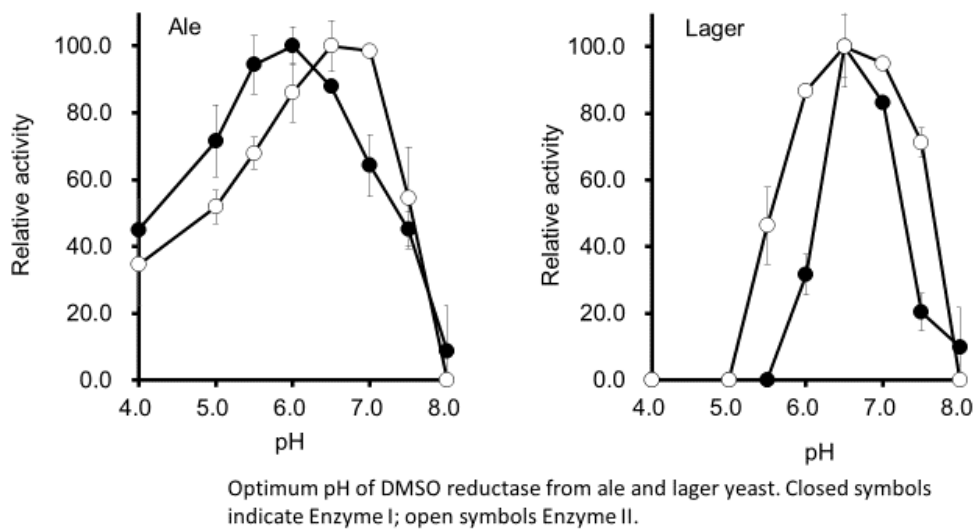
Heat survival ratio of DMSO reductase I and II from ale yeast

Fig.11b



Heat survival ratio of DMSO reductase I and II from lager yeast

Fig.12



Optimum pH of DMSO reductase from ale and lager yeast. Closed symbols indicate Enzyme I; open symbols Enzyme II.

Fig.13

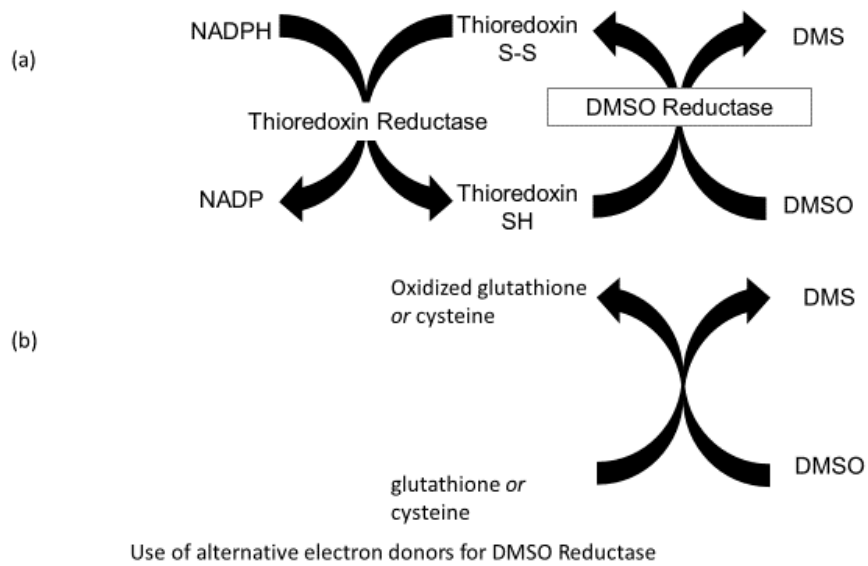
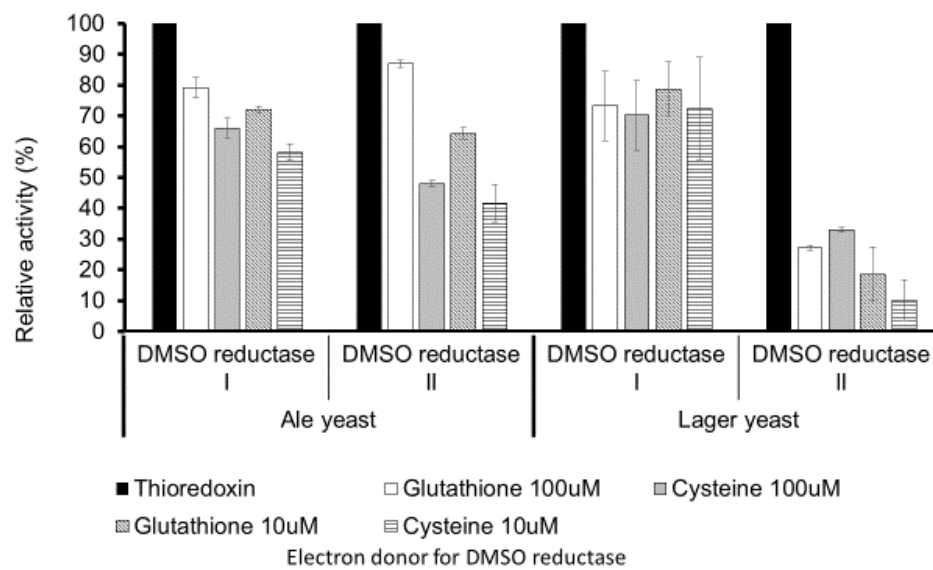


Fig.14



The ability of various electron donors to service DMSO Reductase

